SEPARATION OF BIOLOGICAL MATERIALS IN MICROGRAVITY

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ABSTRACT

Partition in aqueous two phase polymer systems is a potentially useful procedure in downstream processing of both molcular and particulate biomaterials. The potential efficiency of the process for particle and cell isolations is much higher than the useful levels already achieved. Space provides a unique environment in which to test the hypothesis that convection and settling phenomena degrade the performance of the partition process. The intial space experiment in a series arrived at testing this hypothesis is described.

Partition in Two Phase Aqueous Systems

Biotechnology, and the basic research on which it is based, depends heavily upon effective separation and purification processes. Downstream processing is frequently the major cost component in the production of a wide variety of fermentation products. This is dramatically illustrated in Figure 1, taken from Dwyer (1), in which it is seen that the selling costs of a whole range of biotechnological materials are inversely proportional to the initial concentrations at which the species in question are found in the fermentation broth. Hence, the development of inexpensive and effective separation procedures is of major concern to the biotechnology industry.

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One such process which is beginning to receive considerable attention in this regard is partitioning in aqueous two phase systems (2). In this technique phase separation is induced in an aqueous solution by the addition of two incompatible polymers, such as dextran and poly(ethylene glycol) (PEG), or one polymer, typically PEG, and a high concentration of a salt such as sodium phosphate. The resulting two phases provide an extremely benign environment for the separation via partition of almost any type of biological material, from polypeptides and macromolecules up to whole, viable cells (2).

The principle biotechnological interest in aqueous phase partitioning at present is its application in the isolation of macromolecules. However, the preparation of pure subpopulations of viable cells from the complex mixtures in which they are found is also a problem in a number of areas of commercial interest, the isolation of insulin-producing cells for the development of an implantable artificial pancreas being one example (3). Such applications require high resolution, gentle procedures, characteristics which are a feature of the partioning process.

It is perhaps worthwhile to point out that there are very few techniques available which are capable of separating cell subpopulations on the basis of properties that are related to the biological function of the cell. Cell types may differ from one another only in the nature or amount of a single surface membrane constituent, for example, a characteristic that would be insufficient to base a separation on using most approaches. One technique, flow cytometry (also known as fluorescence-activated cell sorting), does provide highly specific separations, being able to select out of a population

those bearing a sufficient density of fluorescently labelled antibodies directed against a particular membrane antigen. The throughput is low, however, since cells are sorted one at a time and only one or two surface features can be utilized on any particular population. Viability can be impaired by the sorting procedure, and only one or two subpopulations can be isolated; no equivalent to chromatography - based on the antigen density, for instance - is possible. Were this technique capable of processing much larger numbers of cells efficiently it might be applicable to clinical problems such as the separation of normal stem cells from tumor cells in bone marrow aspirates, for example. A solution to this problem would lead to much greater success in the treatment of some leukemias and other types of hematological cancers.

Thus, a need exists for a cell purification technique that is able to sort large, heterogeneous mixtures of cells on the basis of specific, subtle features of the cell surface. To a certain extent free-flow electrophoresis fulfills this criterion, being capable of spatially distributing a cell population on the basis of the net charge density associated with the cell surface. However, partitioning also meets the above criterion and is a more sensitive, more versatile and simpler technique. Moreover, it is capable of separating cell populations that are functionally distinct but electrophoretically indistinguishable.

Cell partitioning in two phase systems consisting of aqueous solutions of dextran (a neutral polysaccharide) and poly(ethylene glycol) (PEG) has been shown to be capable of isolating cells on the basis of cell surface charge,

hydrophobicity, differential compatibility of the cell glycocalyx with phase polymers and the presence of groups such as antigens and receptors which have a strong affinity for some soluble agent. The bases for these dependencies have been thoroughly reviewed in recent books (2,4). The separations presently obtainable are useful in a wide variety of settings. Nonetheless, it is clear from the work described below that the efficiency of the process is potentially very much higher than currently available.

One of the most powerful and exciting of the the types of separation under development is immunoaffinity partitioning. In this approach antibodies (Abs) directed against an antigen unique to the cell type of interest are derivatized with one of the phase polymers, usually PEG, so that the Ab-PEG complex partitions strongly into the PEG-rich top phase. When the complex binds to the cell of interest, that cell likewise increases its partition into the PEG-rich phase because the PEG coating reduces its surface free energy in that environment while raising it in the dextran-rich phase. At present this technique is successful in isolating species bearing high concentration of specific surface antigens, providing a multiple extraction procedure - counter-current distribution - is employed (5,6). If the efficiency of this technique could be increased it would be of great benefit in both clinical and basic biomedical science!

The effectiveness of partition as a separation procedure resides in the fact that the partition coefficient, K, is sensitive to a variety of cell surface characteristics. Moreover, in many cases K appears to depend exponentially on the relevant surface property, particularly on the cell surface charge. This is in contrast to the linear dependence of electrophoretic mobility on surface

charge density. This relationship accounts in part for the relatively higher sensitivity of CCD over preparative cell electrophoresis.

A strength of the partition approach to cell separation is that the conditions which determine the value of K are mainly under experimental control. Hence, the cell characteristic on which the separation is to be based can be made the dominant determinant of K by appropriate choice of operating conditions. For instance, K can be made sensitive to cell surface charge by including anions such as phosphate, sulfate or citrate in the system. These salts partition slightly unequally in dextran/PEG systems and threfore cause a Donnan potential to appear between the phases (5). This potential can apparently interact with the cell surface potential, since charge-dependent cell partitions are readily observed (5,6).

There is an additional characteristic of these systems recognized as being an important determinant of cell partition, namely the interfacial tension, , between the two liquid phases. If cells adsorb at the liquid interface between the phases, the loss of interfacial area relative to the situation in which no adsorption occurs lowers the free energy of the interface by an amount equal to the area lost times the interfacial tension. Hence, the larger the adsorbed particle or the higher the tension, the greater will be the tendency for particle adsorption to occur at the phase boundary. In the aqueous polymer systems under discussion it is almost universally observed for particles larger than a few hundred Angstroms in diameter that significant adsorption at the phase boundary does occur, to such an extent that partition of particles as large as cells generally takes place between only one of the bulk phases and the phase boundary, leaving the other bulk phase empty. The

liquid interface therefore acts as a separate phase so far as partition is concerned. That not all the cells are adsorbed at the phase boundary is almost certainly due to the very low values of interfacial tension which occur in these systems, typically in the range of 1 to 20 $\mu N\ M^{-1}$ (7). It is well known that as more concentrated systems with higher interfacial tensions are used, partition into either the top or bottom phase decreases nonspecifically and all cell types accumulate in the interface.

The qualitative observations outlined above can be formalized into a thermodynamic theory of partitioning. For thermally distributed particles the partition coefficient, K = (number of cells in bulk phase)/(number of cells in interface), should be determined by the free energy difference, ΔG, for cells located either in suspension in one of the phases or adsorbed at the interface between them according to the Boltzmann distribution:

$$K = \exp(-\Delta G/kT) \qquad [1]$$

ΔG is determined by the factors mentioned briefly above, as may be seen from the definition:

$$\Delta G = -\gamma A(1 - (\Delta\gamma - \sigma^2\Delta\Psi/\gamma)^2/4 \qquad [2]$$

where: A = surface area of cell

$\Delta\gamma = \gamma_{pb} - \gamma_{pt}$

γ_{pb}, γ_{pt} = interfacial free energy per unit area of particle in bottom (γ_{pb}) or top (γ_{pt}) phase

σ = surface charge density of cell

$\Delta\Psi$ = electrostatic potential difference between bottom and top phases.

More conveniently, ΔG may be expressed in terms of the contact angle, θ, between the cell surface and the boundary between the phases when the cell is adsorbed at the interface:

$$\cos\theta = (\Delta\gamma_{wc} - \Delta\psi)/\gamma \qquad [3]$$

Hence,

$$\Delta G = -\gamma A(1 - \cos\theta)^2/4 \qquad [4]$$

Extensive studies have recently been carried out to test this relationship, using contact angle measurements to determine ΔG (10). Briefly, it was found that the distribution of cells was stochastic in nature and depended approximately exponentially on ΔG, as predicted by equation [1]. However, the slopes of plots of ln K vs ΔG, which should equal (-kT) according to equation [1], were very different from the predicted value, indicating an effective temperature for the process some four to five orders of magnitude greater than the actual value. That is, an energy much greater than thermal energies was acting to remove cells from the interface, leaving them in suspension in the top phase. Cells which were at their lowest free energy state adsorbed at the phase boundary were therefore being randomly removed from that location by forces and energies which were non-thermal in origin. Clearly, if this random removal of cells from the interface could be reduced or eliminated partition would more closely approach thermodynamic behaviour and the ability of the process to separate cell types charcterized by only small differences in ΔG would be greatly enhanced.

At present we have no direct information on the source of the randomizing energy which is obviously present in these systems. There would appear to be no reason for the adsorption process itself to exhibit a strong statistical component. Presumably, then, dynamic factors associated with the mechanics of phase demixing, settling and cell sedimentation are responsible.

The partition process is initiated by shaking the phase system, containing cells, until a fine emulsion is formed, characterized by a size scale which is probably small relative to cellular dimensions. This emulsion rapidly coalesces, without noticeable settling, until a visible granularity appears (within a few seconds in some systems). Convection then sets in and strong local flow can be seen within the sample until partial bulk phase isolation is present, the upper and lower phases being separated by a thick "interface" region which decreases in thickness with time (on the order of several minutes) until separation is complete. During this latter stage undisturbed cell sedimentation takes place as well as the settling or buoyant rising of small droplets (order of magnitude of cellular dimensions) of each phase suspended in the other. These droplets may have cells entrained within them or adsorbed at their surfaces. Any of the above processes could introduce randomization into the final partition behaviour through the release of cells adsorbed at the interface by fluid shear stresses or intercellular collisions.

If the source of the randomization could be specifically identified, it ought to be possible to design a separation process that minimizes this influence. Much higher resolution separations would result, with attendant benefits to biomedical investigation and biotechnology. It is towards this goal that our microgravity experiments are directed.

Space Experiments

The above discussion suggests that one way to attempt to make the partition process more closely approach the desired thermodynamic behaviour would be to carry out the process in an environment in which the fluid shear stresses and convection present when the phases are coalescing and settling is strongly reduced. The Space Shuttle provides such an environment, so an initial expriment was carried out on mission 51-D in April 1985. Before this series of experiments was begun it was not at all clear that the two polymer phase systems would demix and become localized in an acceptable length of time. This concern was based on the behaviour of an oil/water emulsion which, although it coalesced and settled rapidly following mixing on the ground, had been found to form a stable emulsion in space (11). However, we were pleased to discover that dextran/PEG phase systems did demix reasonably rapidly in a reduced gravity environment.

The experiment flown was an extremely simple one, consisting of a series of small chambers milled into a plexiglass block and covered by plexiglass sheets on the front and back to allow observation of the state of the samples. The chambers were filled with a variety of dextran/PEG phase systems and each supplied with a small ball bearing which acted to mix each sample when the box was agitated manually. Following shaking the apparatus was mounted on a light box and photographed at intervals to record the progress of the demixing.

Some representative photographs of four of the systems taken 19 s and 10 min after mixing are shown in Figure 2. The compositions of the phase systems illustrated are as follows:

#1 5% dextran 500 (M ≈ 500,000), 3.5% PEG 8000 (M ≈ 8,000), 0.1M phosphate buffer, pH 7.2

#2 5% dextran 500, 4% PEG 8000, 0.1M phosphate

#3 6% dextran 500, 4% PEG 8000, 0.1M phosphate

#4 7% dextran 500, 0.29% PEG 8000, 12% Ficoll, 0.1M phosphate

It is clear that after only 19 s some degree of demixing was present. Ten minutes after agitation large areas of each phase have become localized in three of the systems although that in #3 showed no appreciable demixing, presumably because of the combination of high viscosities and interfacial tension in this more concentrated system.

The other interesting feature seen is the tendency for the PEG-rich phase, dyed dark by including Trypan Blue in each sample, to occupy the regions near the chamber walls, the dextran-rich (light) phase forming spheres in the interiors of the containers. This tendency became even more pronounced at longer times (not shown). This distribution occurs because the PEG-rich phase has a higher affinity for the chamber wall than does the other phase, hence it tends to preferentially wet the boundaries and displace the dextran-rich phase. This result was confirmed by making contact angle measurements with the systems illustrated when they were exposed to plexiglass.

The results of this first space experiment were very encouraging then, as it seems clear that demixing of the phases can occur in times which are compatible with cell separation experiments. The rate at which the demixing occurs evidently depends on the system composition, so it can be controlled

experimentally. Localization of the demixed phases apparently can also be controlled if the wetting of the chamber walls can be manipulated. We are currently developing polymeric coupling reactions that will allow us to vary the contact angle at the container wall at will (12).

The next series of space experiments will be aimed at understanding the factors which determine the demixing rate and at investigating the possibility of utilizing weak electric fields to control the rate of demixing and the location of the separated phases (13). It will then be possible to test our working hypothesis that the reduction of convective and settling phenomena will reduce the effects of non-thermodynamic factors on cell partition and increase the resolution of this already powerful separation technique.

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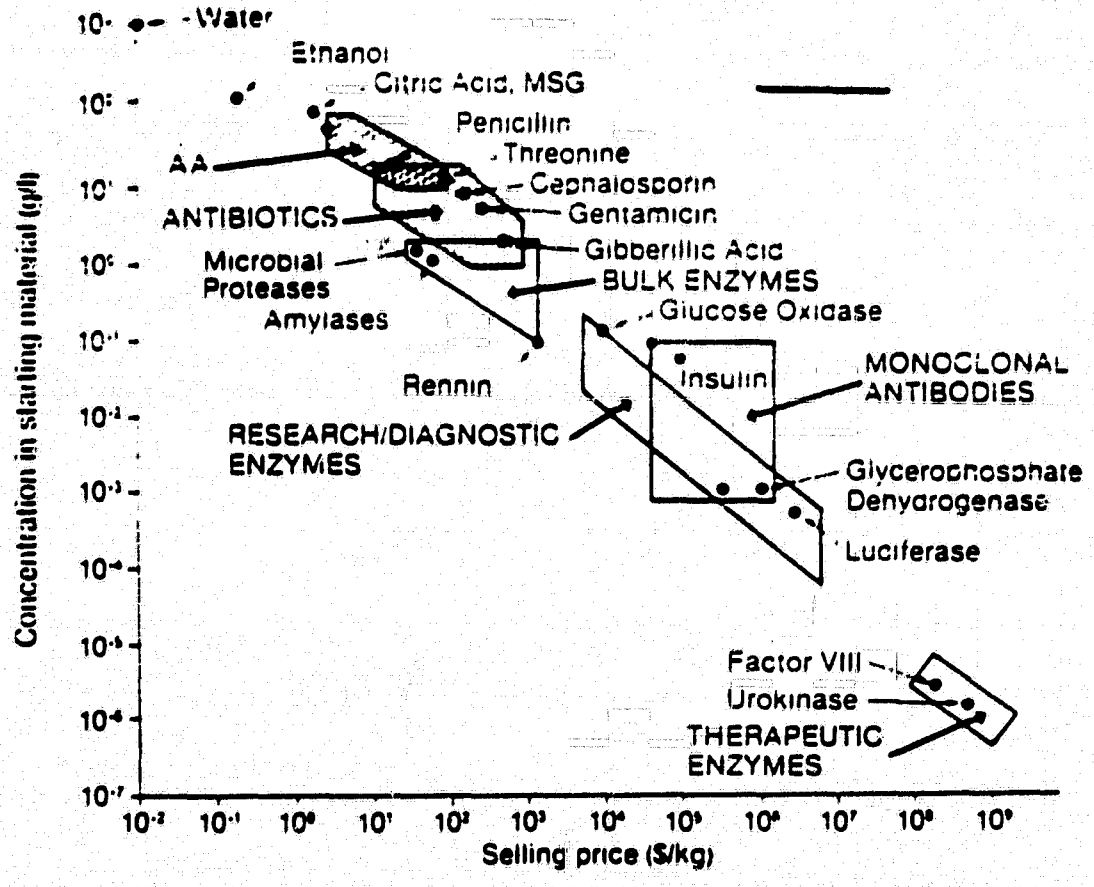
FIGURE LEGENDS

Figure 1. The relationship between starting concentration and selling price for a wide range of biomaterials, taken from Dwyer (1).

Figure 2. The appearance of four different two phase systems 19 seconds (top panel) and 10 minutes (bottom panel) after mixing in a reduced gravity environment. The systems are described in the text.



SEPARATION OF 50 VOLUME % BINARY AQUEOUS IMMISCIBLE
LIQUID DISPERSIONS IN LOW GRAVITY